Journal of Food Protection, Vol. 72, No. 9, 2009, Pages 1812–1820

Planktonic and Biofilm Communities from 7-Day-Old Chicken Cecal Microflora Cultures: Characterization and Resistance to *Salmonella* Colonization†

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MS 09-010: Received 13 January 2009/Accepted 10 April 2009

ABSTRACT

Information implicating bacterial biofilms as contributory factors in the development of environmental bacterial resistance has been increasing. There is a lack of information regarding the role of biofilms within the microbial ecology of the gastrointestinal tract of food animals. This work used a continuous-flow chemostat model derived from the ceca of 7-day-old chicks to characterize these communities and their ability to neutralize invasion by *Salmonella enterica* serovar Typhimurium. We characterized and compared the biofilm and planktonic communities within these microcosms using automated ribotyping and the Analytical Profile Index biotyping system. Eleven species from eight different genera were identified from six culture systems. *Klebsiella pneumoniae* was isolated from all planktonic communities and four of the biofilm communities. Three of the communities resisted colonization by *Salmonella enterica* serovar Typhimurium, two communities suppressed growth, and one community succumbed to colonization. In cultures that resisted colonization, no *Salmonella* could be isolated from the biofilm; in cultures that succumbed to colonization, *Salmonella* was consistently found within the biofilms. This study was one of a series that provided a molecular-based characterization of both the biofilm and planktonic communities from continuous-flow culture systems derived from the cecal microflora of chicks, ranging in age from day-of-hatch to 14 days old. The one common factor relating to successful colonization of the culture was the presence of *Salmonella* within the biofilm. The capacity to sequester the introduced *Salmonella* into the biofilm appears to be a contributing factor to the inability of these cultures to withstand colonization by the *Salmonella*.

Until the 1950s, all poultry production in the United States was ''free range,'' i.e., all poultry was raised outdoors and not segregated by age. When producers began using indoor confinement production, they gained better protection from predators, tighter control of operations, and greater efficiency and overall production. However, producers lost the benefits of protection that newly hatched chicks got from the natural exposure to adult protective microbial communities via contact with adults and their feces *(16)*. To overcome this disadvantage, modern poultry producers have used probiotics or competitive exclusion (CE) products as part of their management practices. A better understanding of the microbial ecology of the poultry gastrointestinal tract (GIT) will help poultry producers to improve their management practices and increase overall profitability and consumer safety.

In 1973, Freter et al. *(17)* were among the first to study gastrointestinal bacterial interactions using a continuous-flow (CF) chemostat, a device for the study of microbes in a controlled and nearly constant environment in which growth conditions are maintained over extended periods by supplying a continuous renewal of nutrients. Using a chemostat the gastrointestinal system can be simulated in order to investigate the community dynamics of microflora *(13, 18, 19, 35)*. Often a stabilized culture has two components, free-floating microorganisms in the liquid medium (the planktonic component) and surface-attached, aggregated microorganisms (the biofilm component). CF chemostat systems have provided researchers with the tools needed to investigate relationships between antibiotics and bacteria *(10, 38)* and to develop effective CE cultures *(23, 34, 44, 55)*. However, the role of biofilms in the CF chemostat culture needs further study.

Bower and Daeschel *(7)* demonstrated that bacterial biofilm formation on food processing surfaces served as an avenue for the development or enhancement of bacterial resistance. Spoering and Lewis *(49)* postulated that one of the survival advantages provided by biofilms is the existence within them of subpopulations of resistant phenotypes. Suci and Tyler *(51)* dubbed these microorganisms ''persisters'' and demonstrated that they represent only a very small fraction of the entire biomass of a culture. Microbes use biofilms as an important strategy for survival in both natural and engineered environments *(27)*. According to Hall-Stoodley et al. *(20)*, ''the realization of the extent to which microbial growth and development occurs on

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surfaces in complex communities has been one of the most subtle advances in microbiology over the past 50 years.'' Researchers now recognize that biofilms are structurally complex and dynamic communities that represent substantial phenotypic diversification; they provide protection from a wide range of environmental challenges, such as UV exposure, metal toxicity, acid exposure, dehydration and salinity, phagocytosis, and several antibiotics and antimicrobial agents *(6, 20)*. Despite interest in the role that biofilms play in bacterial survival and possibly in the development and spread of bacterial antibiotic resistance, ''the majority of biofilms studies only deal with adhesion, the first step of biofilms development and there are almost no microbial studies on biofilms older than 1 week'' *(56)*.

An increased understanding of the factors affecting pathogen colonization in the GIT may help to provide improved control measures. The cecum, with its relatively stable environment, is the site of some of the most diverse microflora communities found within the GIT of poultry *(1, 3, 4, 31)* and serves as an excellent source for the development of CE cultures *(34–36, 39–43, 45)*. Ceca are a primary reservoir for *Salmonella* colonization in young chicks as more than 4 weeks may be required for the cecum to establish a protective native adult microfloral community *(24, 25, 32, 37, 48)*. Numerous researchers have explored the use of CE cultures and probiotics as alternatives to the use of antibiotics in various agricultural situations *(5, 34, 44, 55)*. CF culture systems have been used to develop CE cultures *(23, 34, 44, 55)* and to explore relationships between antibiotics and bacteria *(10, 38)*. However, despite increased information about the importance of bacterial biofilms, their role in the development and maintenance of effective CE cultures is insufficiently understood *(57)*. In this study, we used automated ribotyping to characterize both the planktonic and biofilm communities of CF cultures derived from the cecal microflora of 7-day-old chicks and evaluated the ability of these cultures to resist colonization by *Salmonella*.

MATERIALS AND METHODS

Chickens and cecal material collection. German Lohmann Selected Leghorn (LSL) layer chicks (Hy-Line International, Bryan, TX) were obtained on day of hatch. Chicks were housed for 7 days in temperature- and light-controlled rooms, provided with fresh water, and fed ad libitum. Chicks were euthanized by CO_2 asphyxiation, then dipped into a 30% Roccal solution (Valley Vet Supply, Marysville, KS). The body cavity of the chick was opened under aseptic conditions, and the ceca were surgically removed. Ceca were placed into sterile 15-ml conical tubes and kept on ice during the collection period. After transfer into an anaerobic chamber, cecal contents were collected, combined, and thoroughly mixed. Six cultures were characterized in this work. Cultures A, B, and C were started from the combined cecal material from five chicks. Cultures D, E, and F were started from the combined cecal material from 147 chicks.

Culture methods. To start each CF culture, we used an aliquot of 1.2 ± 0.12 g of cecal material combined with modified Viande Levure (VL) medium prepared in our laboratory as previously described *(36)*. Cultures were maintained in BioFlo I fermentors (New Brunswick Scientific Co., Edison, NJ) fitted with a 1-liter chemostat vessel. The chemostat vessel, containing 500 ml of modified VL medium, was constantly flushed with a stream of O_2-free CO_2 to maintain anaerobiosis. The modified VL medium was prepared in Pyrex bottles, autoclaved for 1.5 h, and flushed with a stream of O_2-free CO_2 immediately upon removal from the autoclave. The chemostat vessel was inoculated with the cecal material and the culture was incubated under CF conditions with a vessel turnover time of 24 h and a flow rate of 0.8 ml/min. Cultures were allowed to develop undisturbed for 3 weeks before aliquots of the planktonic community were collected for microbial isolation.

Microbial isolation. The following isolation procedures were performed under sterile aerobic and anaerobic conditions, using a series of selective media. Primary aerobic isolation of the planktonic community was performed by streaking a 10-μl aliquot of the culture onto plates, each containing one of the following media: MacConkey agar, m Enterococcus agar, brilliant green agar (BGA), Rogosa agar (all from Fisher Scientific International, Inc., Hampton, NH), or chromogenic E. coli/coliform agar (Hardy Diagnostics, Santa Maria, CA). Plates were incubated for 24 h at 37°C and then examined with a stereoscopic microscope. Representative isolates were selected for subculture based on their phenotypic differences. Subcultured, purified isolates were plated onto tryptic soy agar (TSA) with 5% sheep blood and incubated aerobically for 24 h at 37°C. The resulting colony growth was used for ribotype analysis. Over 300 isolates with both similar and different morphology were randomly selected and subcultured, purified isolates were screened for visual similarity, and duplicates were eliminated. The remaining isolates, over 158, were plated onto TSA with 5% sheep blood and incubated aerobically for 24 h at 37°C. The resulting colony growth was used for ribotype analysis.

Primary anaerobic isolation of the planktonic community was performed by streaking a 10-μl aliquot of the culture onto plates each containing one of the following media: Brucella blood agar, Bacteroides bile esculin agar (both from Anaerobe Systems, Morgan Hill, CA), and Veillonella agar (Fisher Scientific International, Inc.). Plates were incubated anaerobically for 48 to 72 h at 37°C and then examined with a stereoscopic microscope. Representative isolates were selected for subculture based on their phenotypic differences. Over 150 isolates with both similar and different morphology were randomly selected and subcultured, purified isolates were screened for visual similarity, and duplicates were eliminated. The remaining isolates, over 75, were plated onto TSA with 5% sheep blood and incubated aerobically for 24 to 48 h at 37°C. Isolates that grew aerobically were compared phenotypically with other aerobic isolates, and duplicates were discarded. Isolates found to be obligate anaerobes were plated on Brucella blood agar and incubated anaerobically for 48 to 72 h at 37°C. The resulting colony growth was used for ribotype analysis or Analytical Profile Index (API) biotyping system (bioMérieux, Hazelwood, MO) characterization.

After sampling for culture characterization, the culture was then challenged with *Salmonella enterica* serovar Typhimurium, as previously described *(21, 25)*, with sufficient inoculum to ensure a final bioreactor concentration of 10^6 CFU/ml of medium. However, in this work a green fluorescent protein–expressing *Salmonella enterica* serovar Typhimurium (ST-GFP) *(22)* was used. Three cultures containing VL media only were also challenged with ST-GFP to determine the colonization of *Salmonella* in pristine chemostats (controls). The planktonic component was sampled for bacterial quantification prior to challenge and 15 min, 2 h, and 2, 4, 7, 9, 11, 14, 16, 18, and 21

days postchallenge. Beginning on day 4, separate culture samples were enriched by incubation for 24 h at 37°C in tetrathionate broth followed by plating on BGA and incubation for 24 h at 37°C *(2)*. On day 21, after the final planktonic sample was collected, the remaining planktonic component was drained from the bioreactor. The bioreactor was flushed three times with anaerobic normal saline. Then the biofilm community was collected in duplicate from each of five vessel levels within the chemostat, using two sterile cotton swabs per level: (1) bottom of the chemostat, (2) lowest area of the sidewall, (3) midway up the biofilm, (4) under the biofilm interface ring, and (5) biofilm interface ring. After biofilm collection, swabs were immediately placed into anaerobic transport medium tubes (Anaerobe Systems). One swab was used to streak plates for anaerobic isolation, the other for aerobic isolation. Both anaerobic and aerobic isolation and culturing procedures followed the same scheme as described above. All procedures were approved by the Southern Plains Agricultural Research Center Animal Care and Use Committee.

Fluorescent microscopy. After isolation from the chemostat culture by selective culturing on BGA plates, the presence of the challenge bacteria, ST-GFP, was confirmed using a Leica DMLB fluorescent microscope equipped with a 100× oil lens and a fluorescein isothiocyanate filter pack.

Automated ribotyping. Ribotyping was performed on pure cultures from the selective media culturing procedure. A RiboPrinter Microbial Characterization System (DuPont Qualicon, Inc., Wilmington, DE) was used for all ribotype analyses. As prescribed by the manufacturer, aerobes were plated onto TSA with 5% sheep blood and incubated aerobically at 37°C for 24 h to allow the formation of a bacterial lawn. An aliquot was collected from the lawns using the collection device provided by the manufacturer (DuPont Qualicon). The bacteria were suspended in a neutral pH buffer (DuPont Qualicon). Strict anaerobes were plated onto Brucella blood agar (Anaerobe Systems) and incubated anaerobically at 37°C for 48 h. Single colonies were transferred into tubes containing 9 ml of VL medium and incubated at 37°C for 24 to 48 h. Aliquots (1 ml) of the resulting cultures were centrifuged at 14,000 × *g* for 2 min to pellet the bacteria. Each bacterial pellet was resuspended in sterile phosphate-buffered saline to a turbidity level equivalent to a 6.0 MacFarland standard. All bacterial suspensions were heated at 90°C for 10 min and combined with two lytic enzymes (proprietary, DuPont Qualicon). DNA was cleaved using the restriction endonuclease *Eco*RI (DuPont Qualicon). The fragments were separated by gel electrophoresis and analyzed by a modified Southern hybridization blotting technique. The DNA was hybridized with a labeled rRNA operon probe (DuPont Qualicon) derived from *Escherichia coli,* and the bands were detected using a chemiluminescent substrate. The resulting image was captured using a customized charge-coupled device camera, and then electronically transferred to the RiboPrinter Microbial Characterization System database. Each sample lane of data was normalized to a standard marker set. The resulting riboprint patterns were compared to a total of 7,348 individual *Eco*RI patterns, 6,448 in the DuPont database and an additional 900 in an in-house custom database of organisms from livestock and poultry sources. An individual ribopattern match of ≥85% of an existing ribopattern was used to characterize each isolate as a specific organism.

RESULTS

Determination of the bacterial genera and species by the RiboPrinter involves the analysis of both the number and intensity of bands (DNA fragments) obtained from the restriction enzyme cleavage of each isolate to develop a ribopattern. This system is capable of resolving fragments ranging in size from 1 to 50 kb. The cecal-derived CF cultures yielded 11 species from eight genera (Table 1). The ribopatterns of the 11 species isolated from the cultures are shown in Figure 1. There were only slight differences between the number of gram-negative and gram-positive bacteria present in the planktonic and biofilm communities. In general, planktonic communities showed a slightly higher diversity than did biofilm communities. Nine of the 11 species were found in both the planktonic and biofilm culture communities from at least one chemostat.

TABLE 1. *Species isolated from the planktonic and biofilm communities of continuous-flow cultures derived from the cecal microflora of 7-day-old chicks*

		Planktonic	Biofilm
	Gram-positive bacteria		
	Clostridium sporogenes	X	X
LAB[a]	*Enterococcus avium*	X	X
LAB	*E. faecalis*	X	X
LAB	*E. faecium*	X	X
LAB	*E. gallinarium*	—[b]	X
LAB	*Pediococcus acidilactici*	X	X
	Gram-negative bacteria		
	Bacteroides spp.	X	X
	Escherichia coli	X	X
	Klebsiella pneumoniae	X	X
	Proteus mirabilis	X	X
	Pseudomonas aeruginosa	X	—

[a] LAB, lactic acid bacteria.
[b] —, not present in culture.

All of the planktonic communities contained four or more species of bacteria (Table 2). *Klebsiella pneumoniae* was the only species found in all of the planktonic communities. Though cultures A, B, and C were derived from a common pool of cecal material, the planktonic communities that developed exhibited some striking differences. The planktonic community from culture C was the most diverse, containing seven species from six genera; the planktonic community from culture B was the least diverse, containing four species from three genera. *K. pneumoniae* and *Enterococcus faecalis* were common to the planktonic communities of cultures A, B, and C. Cultures D, E, and F were also derived from a common pool of cecal material; however, unlike cultures A, B, and C, the planktonic communities that developed in these cultures were quite similar. Cultures D and E both contained five species; culture F contained six. All three cultures contained *K. pneumoniae, Proteus mirabilis,* and *Bacteroides* spp. Sixty percent of the species isolated from planktonic communities were found in three or more cultures.

All six planktonic communities contained at least two lactic acid bacteria (Table 2). The planktonic communities in cultures B and F were composed of gram-positive and gram-negative bacteria in equal proportions. The percentage of gram-positive bacteria found in the planktonic commu-

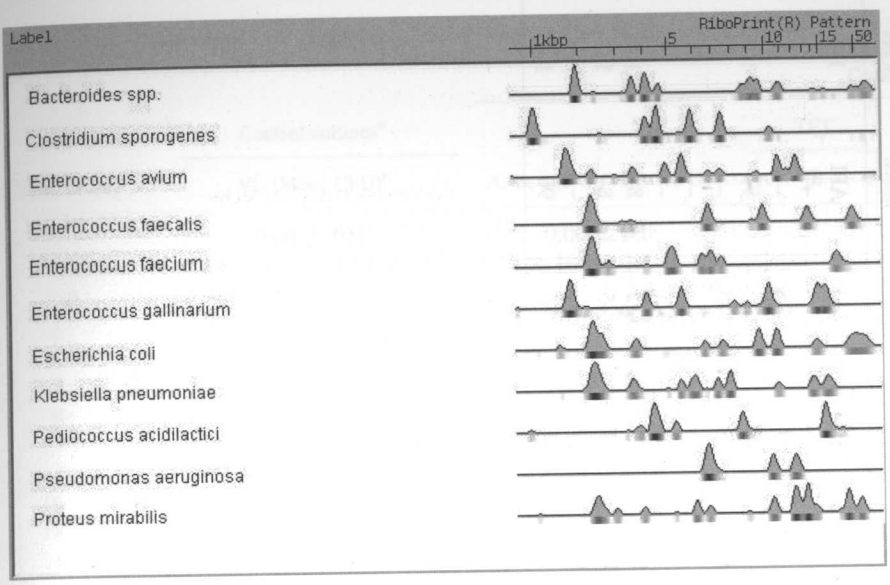

FIGURE 1. *Riboprint patterns for the 11 species of bacteria (*Bacteroides *spp.,* Clostridium sporogenes, Enterococcus avium, E. faecalis, E. faecium, E. gallinarium, Escherichia coli, Klebsiella pneumoniae, Pediococcus acidilactici, Proteus mirabilis, *and* Pseudomonas aeruginosa*) isolated from the CF cultures derived from cecal contents of 7-day-old chicks.*

nities ranged from a low of 40% in cultures D and E to a high of 75% in culture A. *Clostridium sporogenes, Enterococcus avium,* and *Pseudomonas aeruginosa* were each present in only one of the planktonic communities.

Sixty-seven percent of the biofilm communities contained four or more species of bacteria (Table 3). The biofilm community from culture E was the most diverse, containing eight species from six genera; however, there was an even distribution of species between gram-positive and gram-negative bacteria. Culture C had the only completely gram-positive biofilm community. *E. faecalis* and *Pediococcus acidilactici* were both isolated from the biofilm community in 83% of the cultures (Table 3).

C. sporogenes, E. avium, and *Enterococcus gallinarium* were each isolated from only one biofilm community. The percentage of lactic acid bacteria present in biofilm communities ranged from a high of 100% in culture C to a low of 33% in culture B. Interestingly, both cultures A and E each contained five species of lactic acid bacteria, the highest number found in any biofilm community.

ST-GFP was used to challenge chemostats containing modified VL media only (control) and cecal material (Table 4). The presence or absence of ST-GFP within the culture was monitored by growth on selective media, enrichment in tetrathionate broth, and confirmation by fluorescence microscopy (Table 4).

No other *Salmonella* was found in the cultures. ST-GFP rapidly colonized the control chemostats containing media only. The cultures obtained a concentration greater than 10^7 CFU/ml of ST-GFP within 2 days and maintained that concentration for 21 days postinoculation. Upon challenge of chemostats A, B, and C with established cecal communities, ST-GFP concentrations quickly dropped over the first 4 days then continued to drop more slowly over the next week. Chemostats B and C eliminated the ST-GFP from the culture by days 14 and 9, respectively; however, a

TABLE 2. *Characterization of planktonic community species by culture*

	Culture:					
	A	B	C	D	E	F
Bacteroides spp.	—[a]	—	—	API[b]	API	API
Clostridium sporogenes	81[c]/sp[d]	—	—	—	—	—
Enterococcus avium	—	—	—	—	91	—
E. faecalis	92	92	92	96	—	92
E. faecium	—	95	92	—	95	95
Escherichia coli	—	97	88	—	—	—
Klebsiella pneumoniae	97	88	97	92	97	97
Pediococcus acidilactici	85	—	85	85	—	85
Proteus mirabilis	—	—	81/sw[e]	81/sw	81/sw	81/sw
Pseudomonas aeruginosa	—	—	91	—	—	—
ST-GFP[f]	90	—	—	—	85	89

[a] —, not present in culture.
[b] API rapid ID 32 A.
[c] Percent ribotype match for identification.
[d] sp, positive spore test identification confirmation.
[e] sw, positive swarming tests for identification confirmation.
[f] ST-GFP, green fluorescent protein–expressing *Salmonella enterica* serovar Typhimurium.

TABLE 3. *Characterization of biofilm community species by level in culture vessel*

	Culture:											
	A		B		C		D		E		F	
	Species match	Vessel level[a]	Species match	Vessel level	Species match	Vessel level	Species match	Vessel level	Species match	Vessel level	Species match	Vessel level
Bacteroides spp.	—[b]		—		—		API[c]	1	API	1–5	API	1–5
Clostridium sporogenes	81[d]/sp[e]	1–5	—		—		—		—		—	
Enterococcus avium	—		—		—		—		91	1–5	—	
E. faecalis	96	1,2,3,5	96	1–5	96	1,2,3,5	—		96	1–5	96	1–5
E. faecium	95	4,5	—		95	1–5	95	1–5	95	1–5	—	
E. gallinarium	88	5	—		—		—		—		—	
Escherichia coli	—		88	1–5	—		—		88	4,5	—	
Klebsiella pneumoniae	—		85	1–5	—		85	1–5	97	1–5	85	1–5
Pediococcus acidilactici	87	2,4	—		87	5	87	1–5	87	1–5	87	1–5
Proteus mirabilis	82/sw[f]	1–5	—		—		82/sw	1–5	82/sw	3	—	
ST-GFP[g]	88	1–5	—		—		—		91	3–5	90	1–5

[a] 1, bottom of the chemostat; 2, lowest area of the sidewall; 3, midway up the biofilm; 4, under the biofilm interface ring; and 5, biofilm interface ring.
[b] —, not present in culture.
[c] API rapid ID 32 A.
[d] Percent ribotype match for identification of species.
[e] sp, positive spore test for confirmation of species.
[f] sw, positive swarming test for confirmation of species.
[g] ST-GFP, green fluorescent protein–expressing *Salmonella enterica* serovar Typhimurium.

TABLE 4. *Plate counts for control and cecal cultures after* Salmonella *challenge*

Time (days)	Control cultures[a]	Cecal cultures								
			TET[c]				TET		
	VL (Mean CFU)[b]	A, B, and C (Mean CFU)	A	B	C	D, E, and F (Mean CFU)	D	E	F
0	0.00 ± 0.0	0.00 ± 0.0				0.00 ± 0.0			
0.08	$7.69 \times 10^5 \pm 4.62 \times 10^5$	$6.26 \times 10^7 \pm 1.1 \times 10^7$				$1.25 \times 10^7 \pm 2.1 \times 10^7$			
0.2	$1.28 \times 10^5 \pm 1.29 \times 10^5$	$1.45 \times 10^8 \pm 2.5 \times 10^8$				$2.73 \times 10^5 \pm 2.1 \times 10^5$			
2	$1.42 \times 10^8 \pm 3.20 \times 10^7$	$1.60 \times 10^4 \pm 2.8 \times 10^4$				$5.48 \times 10^4 \pm 5.7 \times 10^3$			
4	$3.36 \times 10^7 \pm 1.22 \times 10^7$	$5.09 \times 10^1 \pm 8.5 \times 10^0$	+	+	+	$3.34 \times 10^5 \pm 5.6 \times 10^5$	+	+	+
7	$2.74 \times 10^7 \pm 4.22 \times 10^6$	$1.01 \times 10^2 \pm 1.7 \times 10^2$	+	+	+	$1.79 \times 10^3 \pm 1.5 \times 10^3$	+	+	+
9	$3.59 \times 10^7 \pm 1.86 \times 10^7$	$6.67 \times 10^2 \pm 1.2 \times 10^1$	+	+	−	$4.77 \times 10^2 \pm 3.8 \times 10^7$	+	+	+
11	$1.09 \times 10^7 \pm 2.55 \times 10^6$	0.00 ± 0.00	+	+	−	$9.00 \times 10^1 \pm 7.6 \times 10^1$	+	+	+
14	$3.80 \times 10^7 \pm 1.21 \times 10^7$	0.00 ± 0.00	+	−	−	$2.82 \times 10^1 \pm 2.8 \times 10^1$	−	+	+
16	$1.05 \times 10^7 \pm 1.56 \times 10^6$	0.00 ± 0.00	+	−	−	$2.60 \times 10^1 \pm 3.7 \times 10^1$	−	+	+
18	$1.58 \times 10^7 \pm 6.60 \times 10^6$	0.00 ± 0.00	+	−	−	$1.35 \times 10^2 \pm 2.3 \times 10^2$	−	+	+
21	$2.20 \times 10^7 \pm 5.70 \times 10^6$	0.00 ± 0.00	+	−	−	$2.58 \times 10^3 \pm 4.5 \times 10^3$	−	+	+

[a] All cultures were challenged with 10^6 CFU/ml of ST-GFP.
[b] The average ± standard deviation of three independent control cultures.
[c] The results of overnight enrichment of a planktonic sample of each culture in tetrathionate broth.

small ST-GFP population remained viable in culture A at a level detectable only by enrichment for 21 days. Upon challenge of chemostats D and E with established cecal communities, ST-GFP concentrations steadily dropped over the 3-week period. Chemostat D eliminated the ST-GFP from the culture by day 14; however, a small ST-GFP population remained viable in culture E at a level detectable only by enrichment through day 21. Upon challenge of culture F, ST-GFP concentrations quickly dropped over the first 2 days, then more slowly over the next 2 weeks; however, at day 16 the population of ST-GFP had begun to increase and continued to increase through day 21. No ST-GFP was isolated from the biofilm in cultures B, C, and D, all of which were able to resist *Salmonella* colonization. ST-GFP was isolated from the biofilm in cultures A, E, and F, none of which were able to resist *Salmonella* colonization.

DISCUSSION

The planktonic microbial communities in cultures from the ceca of adult chickens have been characterized by several researchers *(5, 14–17, 38)*. However, their studies did not consider the biofilm component of these cultures. This study examines bacterial communities derived from the ceca of 7-day-old chickens and characterizes both the planktonic and biofilm components of these communities, using automated ribotyping. It is recognized that the cecal microflora can be significantly influenced by environmental factors; therefore, the data presented here should be regarded as case specific. In this study, replicate aliquots from different sources of cecal material were used to start the cultures. Even though 29 times more birds were used to start cultures D, E, and F, those cultures showed only minor differences from cultures A, B, and C in community composition complexity. Note that in each set of cultures started from the same source, all of the planktonic and biofilm communities within the replicates were different. In addition, unlike the data of Bradshaw et al. *(8)*, our data showed that the biofilms in these systems were not a direct reflection of the surrounding planktonic community. In cultures A, B, and C, *E. faecalis* was the only species common to both communities in all cultures, while in cultures D, E, and F, both *Bacteroides* spp. and *K. pneumoniae* were the common species.

The early posthatch stage is critical for establishment of the gut microbial community, and chicks are notably more vulnerable to *Salmonella* infection at this stage *(44, 45)*. Until birth, the gastrointestinal ecosystem is a relatively sterile environment, devoid of microbial colonization. After hatch, chicks pick up a variety of microbes from their surroundings that colonize the niches of the GIT. There is a succession of colonization until all habitats are occupied by climax communities *(30, 46)*. Chicks in production facilities are at risk of a delayed development of their intestinal flora because of sanitized conditions and restricted access between generations. In healthy adult birds, a nonindigenous microbe, such as *Salmonella,* is usually transitory because available niches are occupied by climax communities of indigenous microbes that resist new colonization. However, new microbes may establish in a habitat not yet occupied or one vacated after some perturbation. The economic impact of such colonization and of subsequent human illness from foodborne pathogens in processed foodstuffs can be substantial *(47)*.

The susceptibility of the host to invasion and the resulting long-term retention of a pathogen changes with age and with the volume and complexity of indigenous GIT microbes. Adult birds have a complex community structure better able to fend off enteropathogen colonization, while neonate chicks lack this community sophistication and are more susceptible to infection. In this study, we evaluated the composition of a culture from 7-day-old chick ceca and the success of colonization by *Salmonella* within the established culture. Our cultures contained fewer microbes in comparison to cultures initiated from older birds *(48–50)*.

Researchers have shown that volatile fatty acids (VFAs) produced by lactic and propionic acid bacteria within the ceca of chickens play a key role in providing protection from *Salmonella* invasion *(26, 48, 51)*. van der Wielen et al. *(53)* showed that the bacteriostatic effects of VFAs result in the reduction or elimination of *Salmonella* from the intestinal microflora. Further, Corrier et al. *(12)* demonstrated a direct correlation between elevated levels of propionic acid and decreased numbers of *Salmonella*. Additionally, reduced levels of these VFAs, while able to lower the biomass of *Salmonella,* were incapable of eliminating it *(54)*. However, in this study, the increased number of lactic acid–producing bacterial species within the cultures did not correlate to control of *Salmonella* numbers. We do not know the concentration of these bacteria within the culture; therefore, their influence cannot be discounted *(11)*. The colonization process within the GIT is influenced by age, the environment, and by changes exerted by the make-up of the colonizers themselves. The characterization of the composition of microbes in 7-day-old birds was more clear-cut because the diversity of bacterial species was less than that found in adult birds. In an effort to understand which microbes prevent pathogen colonization, it is important to know which constituents are not essential.

Two cultures (A and C) had several species that segregated into either the biofilm or planktonic portion. Adjustments in population density, diversity, and adhesion occurring during the culturing period could play a part in this phenomenon. Further, the relatively long residence time in rich media could have selected for fast growing species in the planktonic community while slower growing species were sequestered into the biofilm. The variety observed among bacteria species found at the different levels of the biofilm can be attributed to environmental factors, such as surface and interface properties, the composition of the microbial community, and the hydrodynamics of the vessel *(15)*. Additionally, most biofilms exist as patches of cell aggregates, not monolayers, and thus exhibit some level of heterogeneity *(15)*.

Biofilms are a source of long-term contamination by both *Salmonella* and *Campylobacter jejuni (28, 29, 52)* and are implicated as a factor that enhances the resistance and virulence of *Salmonella (28, 33, 53, 54)*. Biofilms may be an important strategy of microbial survival in both natural and engineered environments and may contribute to the development of bacterial resistance *(7, 27)*. Biofilm formation and persistence has profound implications, because microorganisms growing as biofilms are significantly less susceptible to host defenses than are planktonic forms *(9)*. Singh et al. *(47)* state that biofilms are distinct, matrix-encased communities specialized for surface persistence of bacteria. Further, biofilms are notorious for resisting destruction by host defense mechanisms; work by Lewis *(30)* demonstrated that a small fraction of persister cells is ultimately responsible for this very high level of resistance. For these reasons, the role of biofilm communities in the development and maintenance of model chemostat cultures needs to be examined.

In this study, half of the cultures from 7-day-old chicks were able to resist colonization by *Salmonella*, although it took an average of 12 days. Of the three cultures unable to eliminate the invading *Salmonella,* two were able to suppress growth in the *Salmonella* population over the 3-week period, presumably due to the influence of the resident bacteria. The *Salmonella* population in these cultures remained at a very low level, requiring overnight enrichment of samples to detect the presence of the invader. The final culture succumbed to colonization, as the *Salmonella* population appeared to gain a foothold and began multiplying. Note that ST-GFP was isolated from the biofilm communities only in the three cultures that were unable to withstand colonization by *Salmonella*.

This work is part of a series of experiments conducted in our laboratory that evaluated the ability of cultures started from day-of-hatch, 7-day-old, and 14-day-old chick cecal material to withstand colonization by *Salmonella enterica* serovar Typhimurium *(14, 46)*. Our work clearly shows an incremental increase in the ability of these cultures to resist colonization by *Salmonella*. All of the day-of-hatch and half of the 7-day-old cultures succumbed to colonization by *Salmonella,* while none of the 14-day-old cultures did. The presence of *Salmonella* within the biofilm of the cultures was clearly linked with this susceptibility to infection. It appears that these sequestered persistent *Salmonella* acted as a reservoir for persistent infection of the cultures *(14, 46)*.

These observations strongly indicate that bacteria critical to the composition of the planktonic culture may be sequestered within the biofilm. Thus bacteria critical to the efficacy of CE cultures could also be inhabiting the biofilm. Further research is needed to determine which bacterial species help protect the community from colonization and which bacteria are merely spectators and to ascertain the influence of the sequestered biofilm communities on the planktonic portion of the culture. The planktonic component is the portion often harvested for CE products. This work provides the foundation for the development of less complex, but efficacious CE products that could be produced more economically than current products containing 10 or more bacteria.

ACKNOWLEDGMENTS

The authors thank Melanie Sandera, Douglas Psenick, Reiley Street, Gretchen Jones, and Jane Pishko for their technical assistance and Jay Hinton for the *Salmonella* strain.

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